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Phosphorylation of rat liver inorganic pyrophosphatase by ATP in the absence and in the presence of protein kinase

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Cytoplasmic inorganic pyrophosphatase of rat liver can be phosphorylated by cAMP-dependent protein kinase on a serine residue with a concomitant increase in enzymic activity. Phosphorylation is also observed in the absence of protein kinase, but in this case much higher concentrations of ATP are required and the stability characteristics of the phosphoenzyme resemble those of an acyl phosphate. Kinase-free phosphorylation of the animal inorganic pyrophosphatase, unlike that of microbial pyrophosphatases, does not activate the enzyme. Pyrophosphatase may thus provide a new example of an enzyme whose evolution involves convergence of regulatory phosphorylation mechanisms.

Inorganic pyrophosphatase; Protein phosphorylation; Enzyme activation; Protein kinase, cAMP-dependent; Phosphoserine; Enzyme evolution

1. INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1) hydrolyzes pyrophosphate and controls in this way numerous biosynthetic reactions yielding PP_i as a by-product. This enzyme is synthesized constitutively and regulated at the activity level. Phosphorylation by ATP, precursor of PP_i in many biosynthetic reactions, provides one of the means of this regulation as shown recently for baker's yeast and *Escherichia coli* pyrophosphatases [1,2]. Surprisingly enough, ATP phosphorylates these enzymes in the absence of any protein kinase and at a site other than the active one.

In this paper, we show that an animal pyrophosphatase can also be phosphorylated in a kinase-free manner but without any effect on activity. Significant activation is, however, observed when the phosphorylation is effected by a cAMP-dependent protein kinase, i.e. via a mechanism characteristic of multicellular organisms.

2. MATERIALS AND METHODS

Highly purified pyrophosphatase of rat liver cytoplasm was obtained as described [3]. Molar concentration of this dimeric enzyme was estimated with a dye-binding procedure [4] using the molecular mass of 70 kDa [3]. cAMP-dependent protein kinase of bovine heart was from Sigma, ^{32}P-labelled compounds were from Radiopreparat (Tashkent, USSR) and cellulose and polyethyleneimine cellulose sheets for thin-layer chromatography were from Kodak and Merck, respectively.

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The phosphorylation was accomplished by incubating 30 μg pyrophosphatase at 25–30°C in 0.1 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$, 3 mM dithioerythritol, 10 μM ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetate and [γ-^{32}P]ATP, [α-^{32}P]ATP or $^{32}P_i$ at indicated concentrations (~2 × 10^4 cpm/nmol). When specified, the incubation mixture also contained 10 μM cAMP and 0.5–5 μg protein kinase. The incubation time was 20 min in the absence of protein kinase and 5–30 min in its presence. Unbound label was separated by centrifugal gel filtration [5] using 1.5-ml columns of Sephadex G-50 fine. The eluate was assayed for radioactivity, protein content and pyrophoshatase activity. Controls lacking pyrophosphatase and/or protein kinase were run in parallel.

Hydrolysis of the phosphorylated enzyme with 5.7 M HCl was carried out for 3 h at 110°C. The reaction products were subjected to thin-layer chromatography on cellulose sheets as described by Neufeld et al. [6].

The stability of the phosphorylated enzyme was characterized by incubating it for 1 h at 55°C in the following buffers (0.2 mM): glycine-HCl (pH 1.9), ammonium acetate (pH 4.5), Tris-HCl (pH 7.6) and glycine-NaOH (pH 12.5) or for 15 min at 37°C in 0.2 M Tris-HCl (pH 7.6) buffer containing 0.1 M hydroxylamine. The reaction products were subjected to thin-layer chromatography on polyethyleneimine cellulose in 1.2 M LiCl. The values of R_f for phosphoenzyme and P_i in this system were <0.1 and 0.8, respectively.

The phosphate content of native pyrophosphatase was measured using a Malachite green procedure after digesting the enzyme with $HClO_4$ [7].

Initial rates of PP_i hydrolysis were measured using an automated P_i assay [8]. The reaction was carried out at 25°C in 50 mM Tris-HCl buffer (pH 7.2) containing 2–20 μM PP_i, 0.5 mM $MgCl_2$ and 10 μM ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetate.

3. RESULTS

The incubation of rat liver pyrophosphatase with [γ-^{32}P]ATP in the absence of protein kinase led to incorporation of up to 0.5 mol ^{32}P/mol enzyme (Fig. 1). No labelling was observed with 3 mM [α-^{32}P]ATP in-

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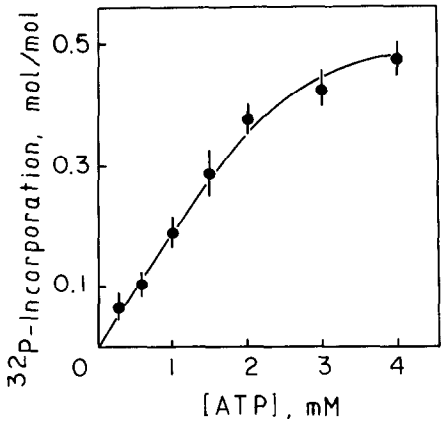

Fig. 1. Degree of pyrophosphatase phosphorylation in the absence of protein kinase as a function of ATP concentration.

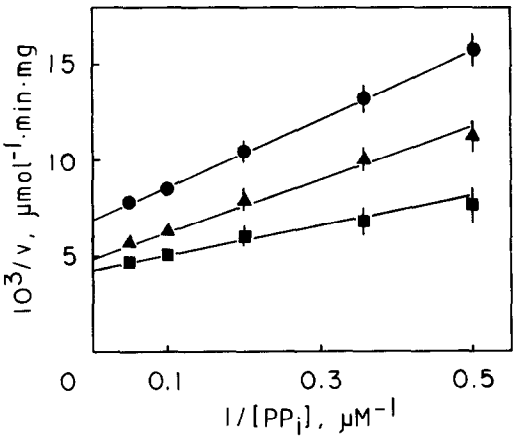

Fig. 2. Lineweaver-Burk plots for the hydrolysis of PP_i by native (●) and protein kinase-phosphorylated pyrophosphatase (▲, ■). Phosphoryl incorporation (mol/mol): ▲, 0.14 ± 0.02; ■, 0.51 ± 0.03.

dicating that the adenosine moiety is not bound to the enzyme. The phosphorylation did not affect the activity of rat liver pyrophoshatase with 2–20 μM PP_i.

The hydrolysis of the phosphorylated protein with 5.7 M HCl for 3 h yielded a single labelled product, which was identified chromatographically as $^{32}P_i$. The phosphorylated enzyme was relatively stable at pH 4.5 and 7.6 (approximately 50% hydrolyzed in 1 h) but disappeared completely yielding $^{32}P_i$ when incubated at pH 1.9 or 12.5. This stability profile is typical of acyl phosphates, which was further corroborated by the finding that treatment with hydroxylamine for 15 min at pH 7.6 led to complete dephosphorylation.

No label was incorporated into pyrophosphatase when it was incubated with 5–20 mM $^{32}P_i$.

Lack of activity change upon kinase-free phosphorylation led to a suggestion that pyrophosphatase activity in higher organisms may be controlled by intercellular signals, for instance, through changes in cAMP level. Therefore, the effect of cAMP-dependent protein kinase on rat liver pyrophosphatase was investigated. The kinase-free reaction could be neglected in the experiments reported below since the ATP concentration as low as 0.1 mM was used (cf. Fig. 1). It was observed that the protein kinase did catalyze the phosphorylation of the pyrophosphatase. The extent of ^{32}P incorporation increased during the first 5–10 min of the incubation and levelled off thereafter because of kinase inactivation. The amount of the bound label was nearly proportional to the concentration of protein kinase added and approached 0.5 mol/mol for 0.15 μg protein kinase/mg pyrophosphatase.

The activity of pyrophosphatase increased considerably upon kinase-mediated phosphorylation, the effect being mostly due to a change in maximal velocity (Fig. 2). Treatment of the phosphorylated enzyme with 5 IU/ml calf intestinal alkaline phosphatase for 3 h (pH 7.2, 22°C) eliminated the activation. At the same time, alkaline phosphatase did not affect the activity of native pyrophosphatase.

The acid hydrolysate of the enzyme phosphorylated with protein kinase was found to contain phosphoserine (Fig. 3), in accord with the substrate specificity of the protein kinase used.

No bound P_i was found in native pyrophosphatase with the colorimetric procedure ruling out a possibility that the observed incorporation of ^{32}P is due to its exchange with preexisting phosphate on the enzyme.

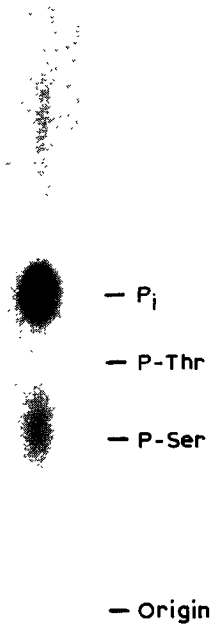

Fig. 3. An autoradiogram illustrating chromatographic identification of phosphoserine in the acid hydrolysate of pyrophosphatase phosphorylated by cAMP-dependent protein kinase.

4. DISCUSSION

Phosphorylation is the most common means of protein modification in a living cell and is generally classified as catalytic, i.e. occurring transiently in enzyme catalysis [9], and regulatory [10]. There is also so-called 'silent' phosphorylation [10] with yet unknown function. Rat liver pyrophosphatase can thus undergo both regulatory and 'silent' phosphorylation, depending on whether protein kinase is present or not.

The two reactions involve different amino acid residues as evidenced by differences in the stability properties of the phosphorylated proteins. In the case of the kinase-free reaction, indirect data suggest that a Glu or Asp residue is involved while protein kinase modifies a Ser one. It should be noted that formation of a covalent bond in the kinase-free reaction has not been shown unequivocally for this enzyme but NMR and chemical data obtained for similar reactions with yeast and *E. coli* pyrophosphatases [1,11,12] leave little doubt about that.

Kinase-free phosphorylation has been reported previously for baker's yeast and *E. coli* pyrophosphatases [1,2] and several other proteins [13–15]. The amino acid residues modified include Ser, Thr, Tyr [13,14], His [12] and Asp (Glu) [1,15]. These reactions illustrate two basic features of protein phosphorylation – spontaneity and diversity, which result from the availability of ATP and other high-energy compounds, on one hand, and the presence of numerous nucleophilic groups in proteins, on the other. The kinase-dependent phosphorylation is no less diverse but more controllable.

These features of protein phosphorylation provide an opportunity to obtain similar functional effects by different means in different organisms. Pyrophosphatase provides a striking example of such convergence in regulatory phosphorylation mechanisms. Both yeast and *E. coli* enzymes are activated by kinase-free phosphorylation, which involves, however, different amino acid residues [1,11,12]. Rat liver pyrophosphatase is also phosphorylated in a kinase-free manner but this reaction is 'silent'. The activation of this enzyme is achieved through a kinase-mediated reaction which involves still another phosphoryl acceptor. So far, convergence of regulatory phosphorylation mechanisms has been demonstrated for phosphorylases [16] and fructose-1,6-bisphosphatases [17], and the present data show that it may be more common for evolution than was realized before.

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